[Translation]

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[Title of the Invention]

Process for Preparation of Nucleoside•Phosphorylase by Recombinant DNA Technique

[Synopsis]

[Object]

The invention has an object of preparing a large amount of nucleoside•phosphorylase by the recombinant DNA technique intending to obtain possibility of efficient synthesis of a nucleoside analogues using nucleoside•phosphorylase.

[Constitution]

The present invention relates to a process for preparation of DNA molecules containing a nucleoside•phosphorylase-structured gene, to preparation of a nucleoside•phosphorylase by the recombinant DNA technique using the same, and to a process for preparation of nucleoside in which the cultivated product, the microbe biomass or the processed product thereof obtained by this method method is used as the enzyme source.

[What is claimed is]

[Claim 1]

A DNA molecule containing a purine nucleoside·phosphorylase-structured gene which codes the amino acid sequence of following formula (I) derived from thermophilic bacteria belonging to bacillus genus

[Formula I]

10 20
MetAsnArgThrAlaIleGluGlnAlaAlaGlnPheLeuLysGluLysPheProThrSer
30 40
ProGlnIleGlyLueIleLeuGlySerGlyLeuGlyValLeuAlaAspGluIleGluGln
50 60
AlaIleLysIleProTyrSerAspIleProAsnPheProValSerThrValGluGlyHis
70 80
AlaGlyGlnLeuValTyrGlyGlnLeuGluGlyAlaThrValValValMetGlnGlyArg
90 100
PheHisTyrTyrGluGlyTyrSerPheAspLysValThrPheProValArgValMetLys
110 120
AlaLeuGlyValGluGlnLeuIleValThrAsnAlaAlaGlyGlyValAsnGluSerPhe
130 140
GluProGlyAspLeuMetIleIleSerAspHisIleAsnAsnMetGlyGlyAsnProLeu
150 160
IleGlyProAsnAspSerAlaLeuGlyValArgPheProAspMetSerGluAlaTyrSer
170 180
LysArgLeuArgGlnLeuAlaLysAspValAlaAsnAspIleGlyLeuArgValArgGlu
190 200
GlyValTyrValAlaAsnThrGlyProAlaTyrGluThrProAlaGluIleArgMetIle
210 220
ArgValMetGlyGlyAspAlaValGlyMetSerThrValProGluValIleValAlaArg
230 240
HisAlaGlyMetGluValLeuGlyIleSerCysIleSerAsnMetAlaAlaGlyIleLeu
250 260
AspGlnProLeuThrHisAspGluValIleGluThrThrGluLysValLysAlaAspPhe
270
LeuArgPheValLysAlaIleValArgAsnMetAlaLysAsn

(I)

[Claim 2]

The DNA molecule according to Claim 1 which contains an SD sequence in the upstream of the purine nucleoside-phosphorylase-structured gene

[Claim 3]

A DNA molecule containing pyrimidine nucleoside-phosphorylase-structured gene which codes the amino acid sequence the following formula (II) derived from thermophilic bacteria belonging to bacillus genus.

[Formula 2]

10 20

MetArgMetValAspLeuIleGluLysLysArgAspGlyHisAlaLeuThrLysGluGlu

30 40

IleGlnPheIleIleGluGlyTyrThrLysGlyAspIleProAspTyrGlnMetSerAla

50 60

LeuAlaMetAlaIlePhePheArgGlyMetAsnGluGluGluThrAlaGluLeuThrMet

70 80

AlaMetValHisSerGlyAspThrIleAspLeuSerArgIleGluGlyIleLysValAsp

90 100

LysHisSerThrGlyGlyValGlyAspThrThrThrLeuValLeuGlyProLeuValAla

110 120

SerValGlyValProValAlaLysMetSerGlyArgGlyLeuGlyHisThrGlyGlyThr

130 140

IleAspLysLeuGluSerValProGlyPheHisValGluIleThrAsnAspGluPheIle

150 160

AspLeuValAsnLysAsnLysIleAlaValValGlyGlnSerGlyAsnLeuThrProAla

170 180

AspLysLysLeuTyrAlaLeuArgAspValThrAlaThrValAsnSerIleProLeuIle

190 200

AlaSerSerIleMetSerLysLysIleAlaAlaGlyAlaAspAlaIleValLeuAspVal

210 220

LysThrGlyValGlyAlaPheMetLysAspLeuAsnAspAlaLysAlaLeuAlaLysAla

230 240

MetValAspIleGlyAsnArgValGlyArgLysThrMetAlaIleIleSerAspMetSer

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【式3】

[Formula 3]

[Formula 3]

250 260
GlnProLeuGlyTyrAlaIleGlyAsnAlaLeuGluValLysGluAlaIleAspThrLeu
270 280
LysGlyGluGlyProGluAspPheGlnGluLeuCysLeuValLeuGlySerHisMetVal
290 300
TyrLeuAlaGluLysAlaSerSerLeuGluGluAlaArgHisMetLeuGluLysAlaMet
310 320
LysAspGlySerAlaLeuGlnThrPheLysThrPheLeuAlaAlaGlnGlyGlyAspAla
330 340
SerValValAspAspProSerLysLeuProGlnAlaLysTyrIleIleGluLeuGluAla
350 360
LysGluAspGlyTyrValSerGluIleValAlaAspAlaValGlyThrAlaAlaMetTrp
370 380
LeuGlyAlaGlyArgAlaThrLysGluSerThrIleAspLeuAlaValGlyLeuValLeu
390 400
ArgLysLysValGlyAspAlaValLysLysGlyGluSerLeuValThrIleTyrSerAsn
410 420
ArgGluGlnValAspAspValLysGlnLysLeuTyrGluAsnIleArgIleSerAlaThr
430
ProValGlnAlaProThrLeuIleTyrAspLysIleSer

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[Claim 4]

The DNA molecule according to Claim 3 which contains an SD sequence in the upstream of pyrimidine nucleoside-phosphorylase-structured gene.

[Claim 5]

A recombinant vector built up by integration of the DNA molecule according to Claim 1 and/or Claim 3 in the downstream of the controlling signal for expression of a vector which is capable of replication in the cell.

[Claim 6]

The recombinant vector according to Claim 5 which

contains at least a promoter the expression-controlling signal of which functions in Escherichia coli.

[Claim 7]

A cultivated product which contains a microbe biomass which retains nucleoside•phosphorylase derived from thermophilic bacteria in which the relevant enzyme has been generated by cultivation of a transformant transformed by the recombinant vector according to Claim 5

[Claim 8]

A microbe biomass which retains nucleoside•phosphorylase derived from thermophilic bacteria isolated from the cultivated product according to Claim 7, or a processed product thereof.

[Claim 9]

A process for preparation of nucleoside characterized in that, in the process in which a base donor, a sugar-residue donor and a phosphoric acid donor are reacted using an enzyme preparation which contains a nucleoside•phosphorylase to form an N-glicoside linkage between the base segment of the base donor and the sugar segment of the sugar-residue donor, the product according to Claim 7 or Claim 8 is used as the enzyme preparation containing a nucleoside•phosphorylase.

[Detailed description of the invention]

[0001]

[Field of application in industry]

The present invention relates to a process for preparation of nucleoside•phosphorylase derived from thermophilic bacteria belonging to bacillus genus by the

technique of recombined DNA and to application of the enzyme obtained through such a process to production of nucleoside.

[0002]

[Prior art]

Chemotherapeutic agents of nucleic acid type are used in various use fields such as antineoplasma, immunosuppression, antivirus and so forth. Also, the antivirus action of nucleoside analogues is attracting attention with epidemy of AIDS in recent years to bring about synthesis of various nucleoside analogues which are subjected to examination of the antiviral activity thereof ("Kagaku-to-Seibutsu", Vol. 27, No. 6, p.356～p.366). Although many of these nucleoside analogues have been hitherto synthesized chemically, in the currency of elucidation of the fact that various nucleoside analogues can be efficiently synthesized by utilizing nucleoside•phosphorylase derived from microorganisms, utilization of such an enzyme has come to be regarded as an important means for synthesis of a nucleoside analogue ("Hakko to Kogyo", Vol. 39, No. 10, p.927～p.937).

[0003]

In general, microorganisms are advantageous as the source for preparation of enzymes from the viewpoint of operability and economy. It has been confirmed that nucleoside•phosphorylases exist in various organisms including animals, microorganisms etc. and the enzymological characteristics of some of them have been reported after isolation and refining thereof. For instance, existence of nucleoside•phosphorylase has been confirmed also with respect to Bacillus

stearothermophilus as a kind of thermophilic bacteria belonging to bacillus genus. That is to say, both of purine nucleoside• phosphorylase (E.C.2,4,2,1.) and pyrimidine nucleoside• phosphorylase (E.C.2,4,2,2.) have been isolated from such microorganisms and refined for reporting various characteristics thereof (J. Biol. Chem., 244, 3691 (1969); Agric. Biol. Chem., 53, 2205 (1989); and Agric. Biol. Chem., 53, 3219 (1989)). Synthesis of a nucleoside analogue using such enzymes have been also reported (Agric. Biol. Chem., 53, 197-202 (1989); Japanese Patent Laid-Open Publication Sho 56-166199; Japanese Patent Laid-Open Publication Sho 56-164793; and Japanese Patent Laid-Open Publication Hei 1-320995).

[0004]

Yamanouchi et al. have found out Bacillus stearothermophilus, TH6-2 strain which contains nucleoside• phosphorylases (purine nucleoside•phosphorylase and pyrimidine nucleoside•phosphorylase) having thermotolerance and high specific activity from thermophilic bacteria belonging to bacillus genus and have succeeded in isolation of a nucleoside• phosphorylase from this microbe strain (International Patent Laid-Open Publication WO90/10080; "Nippon Nogei Kagaku Kai-shi", Vol. 63, No. 3, (Lecture Abstracts for 1989 Convention), p.283).

[0005]

[Problems to be solved by the invention]

Although the above enzymes found out by Yamanouchi et al. are extremely excellent enzymes, in the cases of using the microbe biomass per se of the microorganisms as the enzyme source for nucleoside preparation, the enzyme is released in the

reaction medium due to autolysis accompanied with spore formation inducing disadvantages that continuous synthesis of nucleoside is unfavorably influenced or that synthesis or purification of nucleoside is hindered by flowing out of the microbe biomass accompanied with bacteriolysis. Furthermore, it is necessary to continually recognize that, as a common problem in the cases of using a microbe biomass of microorganisms as an enzyme source, there exists a problem that various enzymes included in the microbe biomass induce side reactions such as disintegration of the stroma or the product bringing about decrease of the product yield.

[0006]

Although a method of using a preparation of purified enzyme as the enzyme source has been taken into consideration in order to solve the problems in the method of using such a microbe biomass of microorganisms as an enzyme source, it has been a matter of difficulty in fact to collect the nucleoside• phosphorylase from the microbe biomass in a high yield since bacillus stearothermophilus has decomposing activity against protein, nucleoside•phosphorylase is produced only in little amount and the refining operation of the enzyme is troublesome.

[0007]

Yamanouchi has carried out cloning of Escherichia coli with a DNA fragment containing a gene for coding the nucleoside• phosphorylase derived from bacillus•stearothermophilus as the first step for overcoming the above problem to find out a fact that the relevant enzyme can be produced by Escherichia coli. (Japanese Patent Laid-Open Publication Hei 4-4882).

However, the production amount of nucleoside•phosphorylase by the recombinant Escherichia coli created by the method is equal to or less than the production amount thereof by the thermophilic bacteria so that, even if such bacteria are used as the enzyme source for nucleoside preparation, such a measure has been by no means bearable in practical application.

[0008]

Means to solve the problem]

The inventors have carried out extensive studies with an object of solving the above problem consequently to succeed in analyzing the primary structure of the DNA for coding the nucleoside phosphorylase derived from bacillus• stearothermophilus and, on the basis of the result of this analysis, in mass production of the enzyme by Escherichia coli applying the recombinant DNA technique thus to complete the present invention.

[0009]

That is to say, the present invention relates to DNA molecules which contain a purine nucleoside•phosphorylase-structured gene for coding the amino acid sequence of the following Formula (I) derived from thermophilic bacteria belonging to bacillus genus.

[0010]

[Formula 4]

10 20

MetAsnArgThrAlaIleGluGlnAlaAlaGlnPheLeuLysGluLysPheProThrSer

30 40

ProGlnIleGlyLueIleLeuGlySerGlyLeuGlyValLeuAlaAspGluIleGluGln

50 60

AlaIleLysIleProTyrSerAspIleProAsnPheProValSerThrValGluGlyHis

70 80

AlaGlyGlnLeuValTyrGlyGlnLeuGluGlyAlaThrValValValMetGlnGlyArg

90 100

PheHisTyrTyrGluGlyTyrSerPheAspLysValThrPheProValArgValMetLys

110 120

AlaLeuGlyValGluGlnLeuIleValThrAsnAlaAlaGlyGlyValAsnGluSerPhe

130 140

GluProGlyAspLeuMetIleIleSerAspHisIleAsnAsnMetGlyGlyAsnProLeu

150 160

IleGlyProAsnAspSerAlaLeuGlyValArgPheProAspMetSerGluAlaTyrSer

170 180

LysArgLeuArgGlnLeuAlaLysAspValAlaAsnAspIleGlyLeuArgValArgGlu

190 200

GlyValTyrValAlaAsnThrGlyProAlaTyrGluThrProAlaGluIleArgMetIle

210 220

ArgValMetGlyGlyAspAlaValGlyMetSerThrValProGluValIleValAlaArg

230 240

HisAlaGlyMetGluValLeuGlyIleSerCysIleSerAsnMetAlaAlaGlyIleLeu

250 260

AspGlnProLeuThrHisAspGluValIleGluThrThrGluLysValLysAlaAspPhe

270

LeuArgPheValLysAlaIleValArgAsnMetAlaLysAsn

(I)

[0011]

Further, the present invention relates to DNA molecules which contains a pyrimidine nucleoside•phosphorylase-structured gene for coding the amino acid sequence of the following Formula (II) derived from thermophilic bacteria belonging to bacillus genus.

【0012】

[Formula 5]

10 20
MetArgMetValAspLeuIleGluLysLysArgAspGlyHisAlaLeuThrLysGluGlu
30 40
IleGlnPheIleIleGluGlyTyrThrLysGlyAspIleProAspTyrGlnMetSerAla
50 60
LeuAlaMetAlaIlePhePheArgGlyMetAsnGluGluGluThrAlaGluLeuThrMet
70 80
AlaMetValHisSerGlyAspThrIleAspLeuSerArgIleGluGlyIleLysValAsp
90 100
LysHisSerThrGlyGlyValGlyAspThrThrThrLeuValLeuGlyProLeuValAla
110 120
SerValGlyValProValAlaLysMetSerGlyArgGlyLeuGlyHisThrGlyGlyThr
130 140
IleAspLysLeuGluSerValProGlyPheHisValGluIleThrAsnAspGluPheIle
150 160
AspLeuValAsnLysAsnLysIleAlaValValGlyGlnSerGlyAsnLeuThrProAla
170 180
AspLysLysLeuTyrAlaLeuArgAspValThrAlaThrValAsnSerIleProLeuIle
190 200
AlaSerSerIleMetSerLysLysIleAlaAlaGlyAlaAspAlaIleValLeuAspVal
210 220
LysThrGlyValGlyAlaPheMetLysAspLeuAsnAspAlaLysAlaLeuAlaLysAla
230 240
MetValAspIleGlyAsnArgValGlyArgLysThrMetAlaIleIleSerAspMetSer

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【式6】

[Formula 6]

250 260
GlnProLeuGlyTyrAlaIleGlyAsnAlaLeuGluValLysGluAlaIleAspThrLeu
270 280
LysGlyGluGlyProGluAspPheGlnGluLeuCysLeuValLeuGlySerHisMetVal
290 300
TyrLeuAlaGluLysAlaSerSerLeuGluGluAlaArgHisMetLeuGluLysAlaMet
310 320
LysAspGlySerAlaLeuGlnThrPheLysThrPheLeuAlaAlaGlnGlyGlyAspAla
330 340
SerValValAspAspProSerLysLeuProGlnAlaLysTyrIleIleGluLeuGluAla
350 360
LysGluAspGlyTyrValSerGluIleValAlaAspAlaValGlyThrAlaAlaMetTrp
370 380
LeuGlyAlaGlyArgAlaThrLysGluSerThrIleAspLeuAlaValGlyLeuValLeu
390 400
ArgLysLysValGlyAspAlaValLysLysGlyGluSerLeuValThrIleTyrSerAsn
410 420
ArgGluGlnValAspAspValLysGlnLysLeuTyrGluAsnIleArgIleSerAlaThr
430
ProValGlnAlaProThrLeuIleTyrAspLysIleSer

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[0013]

Further, the present invention relates to a recombinant vector prepared by integration of a DNA molecule containing the above nucleoside•phosphorylase-structured gene in the downstream of expression-controlling signal of the vector capable of replication in a cell, to a cultivated product containing microbe biomass of microorganisms retaining a nucleoside•phosphorylase derived from thermophilic bacteria in which the relevant enzyme has been generated by cultivation of a transformant transformed by the recombinant vector, and to microbe biomass of microorganisms retaining a nucleoside•

phosphorylase derived from thermophilic bacteria which has been isolated from the cultivated product, or to a processed product thereof.

[0014]

Furthermore, the present invention relates to a process for preparation of nucleoside characterized in that, in the process for preparation of nucleoside by a reaction of a base donor, a sugar-residue donor and a phosphoric acid donor using an enzyme preparation which contains nucleoside• phosphorylase to form N-glicoside linkages between the base segment of the base donor and the sugar segment of the sugar residue donor, the above cultivated product or the above microbe biomass of microorganisms or a processed product thereof is used as the enzyme preparation containing a nucleoside•phosphorylase.

[0015]

The present invention is described below in details. The following terms in this specification are used respectively with the definition shown below. The expression "derived from thermophilic bacteria belonging to bacillus genus" has a meaning of substantial identicalness of the base sequence of a DNA molecule with that of a gene of thermophilic bacteria belonging to bacillus genus but does not necessarily mean that the DNA fragment in the present invention is restricted to an extracted product from thermophilic bacteria belonging to bacillus genus. The phrase "the base sequence is substantially identical" means that, so far as the genetic information as a nucleoside• phosphorylase is maintained, substitution, default and/or addition of several unitary nucleotide (base) may exist.

[0016]

1. DNA molecules containing a nucleoside•phosphorylase-structured gene

The saying "DNA molecule containing a nucleoside•phosphorylase-structured gene derived from thermophilic bacteria belonging to bacillus genus" denotes those containing nucleoside• phosphorylase-structured gene coding the amino acid sequence of the above Formula (I) and/or Formula (II) and the specific base sequence thereof is not restricted in particular. For instance, it is possible to exemplify the DNA molecules specified in the restriction enzyme map shown in Figure 1, more specifically the DNA molecule containing the purine nucleoside•phosphorylase-structured gene, can be exemplified by by that cut off at Ncol and Hpal, the DNA molecule containing the pyrimidine nucleoside•phosphorylase-structured gene can be exemplified by that cut off at Pstl and Hincll and the DNA molecule containing the gene structured by both purine nucleoside•phosphorylase and pyrimidine nucleoside•phosphorylase can be exemplified by that cut off at Ncol and EcoRl, respectively.

[0017]

Such DNA molecules contain an SD sequence at least in the upstream of structural gene in addition to the structural gene of nucleoside•phosphorylase so that they serve the object of present invention in respect of that they notably increase the produced amount of nucleoside•phosphorylase in comparison with the case of using DNA molecules containing only the structural gene. Accordingly, it is essential to use, in the

process of present invention, the DNA molecules containing a base sequence within the range at least from the SD sequence to the ending codon shown in Figures 2～4. In the cases of preparing both enzymes, purine nucleoside•phosphorylase and pyrimidine nucleoside• phosphorylase, it is sufficient to use those the DNA molecules of which include at least one SD sequence in the upstream of structural gene thereof.

[0018]

In Figure 1, "punA" indicates a gene coding the purine nucleoside•phosphorylase (822 bp, coding a polypeptide of molecular weight 29,637 consisting of 274 amino acids) and "pyn" indicates a gene coding the pyrimidine nucleoside• phosphorylase (1298 bp, coding a polypeptide of molecular weight 46,271 consisting of 433 amino acids). In the analysis by the inventors, both of the genes are forming a cluster respectively and have a ribosome binding site which is necessary for beginning translation but have no promoter structure of thermophilic bacteria and no promoter structure capable of functioning by Escherichia coli in the 5' upstream in the proximity thereof.

[0019]

Such DNA molecules can be subjected to cloning from thermophilic bacteria belonging to bacillus genus taking the nucleoside-decomposing activity at high temperatures as the index as seen in the above Yamanouchi's method (Japanese Patent Laid-Open Publication Hei 4-4882) or the like. Otherwise, it is possible to adopt a method, as conventionally well applied, in which a known method is used for determination of the partial

amino acid sequence such as the amino terminals of purified nucleoside•phosphorylase derived from thermophilic bacteria belonging to bacillus genus, or the sequence in part of the amino acid sequence of the above Formula (I) and/or Formula (II) is referred to,an oligonucleotide corresponding thereto is synthesized, and a DNA fragment containing a gene coding the nucleoside•phosphorylase is chosen from the gene bank of thermophilic bacteria belonging to bacillus genus using such an oligonucleotide as a probe. Although the host used for cloning is not restricted, it is appropriate to use Escherichia coli as the host in view of the operableness and the convenience. Further, there is no trouble also in chemical synthesis with use of an ordinary DNA synthesizer referring to Figures 2～4.

[0020]

Usually, even if these fragments are cloned on, e.g., a plasmid vector, such DNA fragments have no promoter or, though having a promoter, efficient functioning in heterogeneous microorganisms cannot be expected in many cases so that it is considered that high expression of coded gene is not induced as usual. Further, in the event of being provided with an extra DNA other than the coding region, the expression thereof may sometimes happen even by read through transcription from the promoter of another gene existing on the plasmid vector as an unfavorable event. Therefore, it is necessary for incarnating high expression of the gene as the object to analyze the base sequence of cloned DNA fragment to specify the coding region of the gene for preparing the recombinant expression vector in which the expression controlling signals (signals for beginning

of transcription and beginning of translation) are combined in the 5' upstream thereof so as to obtain possibility of high expression of the gene in the microbe biomass of microorganisms correspondingly to the host microorganisms. Determination of the DNA base sequence can be made by the ordinary method as exemplified by the possibility of conducting as application of Maxam-Gilbert method (Methods in Enzymology, 65, 499 (1980)), the dideoxy chain terminator method (Methods in Enzymology, 101, 20 (1983)) or the like.

[0021]

2. Recombinant vector

It is preferable to use, as the expression controlling signal used for expression of a large amount of nucleoside• phosphorylase genes in heterogeneous microorganisms, powerful signals for transcription beginning as well as for translation beginning capable of being artificially controlled which make great stride in increase of the expression amount of the nucleoside•phosphorylase genes. Such transcription beginning signals can be exemplified by expression controlling signals for genes such as lac promoter, trp promoter, tac promoter (Proc. Natl. Acad. Sci. USA., 80, 21 (1983); Gene, 20, 231 (1982)), trc promoter (J. Biol. Chem., 260, 3539 (1985)) etc. in the case of using Escherichia coli as the host and by glyceraldehyde-3-phosphate•dehydrogenase (J. Biol. Chem., 254, 2078 (1980)), depressive acidic phosphatase (Nucl. Acids Res., 11, 1657 (1983)) etc. in the case of using an yeast as the host.

[0022]

Although it is possible to use various plasmid

vectors, phage vectors and the like as the vector, it is preferable to use a plasmid vector being replicable in the microbe biomass, having a suitable drug resistant marker and a specific scission site of restriction enzyme and having a high copy number in the microbe biomass. In the case of specifically using Escherichia coli as the host, it is possible to exemplify by pBR322 (Gene, 2, 95 (1975)); pUC18, pUC19 (Gene, 33, 103 (1985)) and so forth. Further, in the case of using an yeast as the host, it is possible to exemplify by YEp13 (ATCC 37115), YEp24 (ATCC37051) and so forth. The methods for preparation of nucleoside•phosphorylase-structured gene, binding of a gene after cloning with an expression controlling signal etc. are well known technology for the technicians in general, particularly for the technicians belonging to the fields of molecular biology or genetic engineering so that it can be practiced depending on the method exemplified by that described in "Molecular Cloning" (edited by Maniatis et al., Cold Spring Harbor, New York (1982)).

[0023]

3. Preparation and cultivation of transformant

Transformation of microorganisms is carried out using the prepared recombinant vector. The microorganisms as the host is not restrictive in particular with a proviso of securing high safety and easy handling. For example, it is possible to use microorganisms conventionally used in DNA recombining operations such as Escherichia coli, yeast and the like. Among them, Escherichia coli is advantageous in vies of handling as well as of synthesis of nucleoside analogues as shown by the usefulness

of, for example, K12 strain, C600 microbe, JM105 microbe, JM109 microbe and the like used in recombinant DNA experiments. The method for subjecting microorganisms to transformation may be appropriately chosen from many methods previously reported correspondingly to the microorganisms used as the host. In the case of using Escherichia coli, for example, as the host, it is possible to subject Escherichia coli to transformation by the method of introducing a plasmid into the microbe biomass at a low temperature after processing by calcium chloride (J. Mol. Biol., 53, 159 (1970)). In the case of using an yeast as the host, it is possible to apply the processes such as the protoplast method (Proc. Natl. Acad. Sci. USA, 75, 1929 (1978)), the alkali metal treating method (J. Bacteriol., 153, 163 (1983)) and so forth.

[0024]

The obtained transformants are subjected to proliferation in a medium in which the relevant microorganisms can be multiplicated and then further to cultivation by inducing of expression of the cloned nucleoside·phosphorylase genes until a large amount of the enzyme is accumulated in the microbe biomass. It is sufficient to carry out cultivation of the transformant by application of an ordinary method with use of a medium containing nutrient sources such as carbon sources, nitrogen sources and the like required for proliferation of the relevant microorganisms. For instance, in the case of using Escherichia coli as the host, it is possible to carry out cultivation using a cultivation medium conventionally used for cultivation of Escherichia coli such as 2xYT medium (Methods in

Enzymology, 100, 20 (1983)), LB medium, M9CA medium (Molecular Cloning, mentioned before) and the like at 20～40 °C of the cultivation temperature with aeration or stirring according to necessity. In the case of using plasmid as the vector, cultivation is carried out by compounding the cultivation medium with an appropriate amount of a drug of suitable antibiotic (such as ampicillin, tetracycline etc. corresponding to the drug-resistant marker of plasmid) in order to prevent falling down of the plasmid during cultivation.

[0025]

In the cases where induction of expression is required for nucleoside•phosphorylase gene in the course of cultivation, expression of the relevant gene is induced by the method ordinarily used for the used promoter. For instance, in the cases of using lac promoter or tac promoter, an appropriate amount of isopropyl-β-D-thiogalactopyranosid (abbreviated as IPTG, hereinafter) as an expression inducing agent is added in the middle course of cultivation. In the cases where the used promoter has transcription activity as the constitution, addition of any expression inducing agent is not required in particular. After expression of the nucleoside•phosphorylase has been induced, cultivation is continued for further several hours for accumulation of a large amount of the gene product in the microbe biomass giving a cultivated product as the enzyme source. The cultivated product thus obtained can be used as an enzyme source for synthesis of a nucleoside analogue.

[0026]

4. Cultivated microbe biomass and processed product thereof

The recombinant bacteria are subjected to recovery of the microbe biomass after cultivation by any treatment such as membrane separation, centrifugal separation or the like. In the cases of using the cultivated microbe biomass as an enzyme source, the collected microbe biomass can be directly used in synthesis of a nucleoside analogue by suspending the collected microbe biomass in a suitable buffer solution. In order to carry out synthesis of an enzymic nucleoside·analogue, it is sufficient to prepare the nucleoside·phosphorylase produced from the recovered microbe biomass. For instance, a cell-free extract solution is obtained from the recovered microbe biomass by suspending in a suitable buffer solution with subsequent crushing of the microbe biomass by a physical measure such as ultrasonic treatment, French press treatment or the like or bacteriolysis such as lysozyme treatment succeeded by removal of the residue of microbe biomass by centrifugation. Since the enzyme exists in excess in the cell-free extract solution, it is also possible to use the cell-free extract solution per se as a preparation. Even in the cases where further refining is required, it is possible to prepare a highly refined enzyme preparation suitable for synthesis of a nucleoside analogue by application of a simple means conventionally used for refining of enzymes such as heat treatment, salting out treatment by ammonium sulfate, dialysis treatment, solvent treatment by, e.g., ethanol, various types of chromatographic treatment and so forth as single application or as a combination of at most 2 kinds of treatments. In the cases of using Escherichia coli as the host microorganisms, almost whole of the protein derived

from Escherichia coli is denatured by subjecting the cell-free extract solution to heat treatment (at 60～80 °C for 1～10 minutes) to allow removal simply by operation of centrifuge so that it is possible to refine an enzyme with very high efficiency.

[0027]

5. Synthesis of nucleoside·analogue

The synthesis of nucleoside·analogue according to the present invention is characterized by the use of above cultivated product, the above cultivated microbe biomass or the processed product thereof and other conditions or procedures are satisfied by following the known method (refer to, for example, International Patent Laid-Open Publication WO90/10080). That is to say, it is possible to practice by setting up the optimum condition of the used enzyme depending on a preliminary test and by carrying out the reaction of the raw compound with the cultivated product, the cultivated microbe biomass or the processed product thereof under this set condition. More specifically, it is possible to carry out by the reaction under the set condition of reaction temperature appropriately selected from 20～95 °C and of pH for reaction appropriately selected from 3～10 using an appropriate buffer solution containing an appropriate amount of inorganic phosphoric acid compounded with nucleoside as the sugar-residue donor and with, e.g., a base analogue as the base donor. The synthesized nucleoside analogue can be purified after the end of reaction,by application of an appropriate combination of the methods usually used as the purification method for substances relating to nucleic acid.

[0028]

[Effect of the invention]

According to the present invention, the above-mentioned problems in the prior art have been solved and it has been made possible to synthesize efficiently nucleoside analogues using a nucleoside•phosphorylase derived from thermophilic bacteria belonging to bacillus genus. Namely, the present invention provides the following advantages.

(a) It is made possible to synthesize efficiently a nucleoside analogue accompanied with few side reactions using a microbe biomass as the enzyme source. In the cases of synthesizing a nucleoside analogue using a microbe biomass of microorganisms belonging to bacillus genus, the side reaction proceeds even in the reactions at a high temperature since the substrates other than nucleoside•phosphorylase or product-decomposing enzymes have thermotolerance. However, almost all of enzymes derived from Escherichia coli are inactivated in the case of conducting the synthesizing reaction at a high temperature using Escherichia coli as the host microorganisms getting rid of almost all of the side reactions. Further, the preceding problem of autolysis is solved by using Escherichia coli as the host microorganisms. Also, since mass production of nucleoside•phosphorylase derived from thermophilic bacteria is carried out in the recombinant bacteria, use of a small amount of microbe biomass is sufficient in use for the synthesizing reaction so that it is made possible to efficiently synthesize in view of economy.

[0029]

(b) Preparation of a large amount of enzyme is made convenient so that it is made possible to put enzymic synthesis of a nucleoside analogue into practical application. Although it has been extremely difficult in the prior art to prepare nucleoside·phosphorylase from thermophilic bacteria belonging to bacillus genus, preparation thereof in a large amount has been extremely convenient by mass production of the enzyme as application of the recombinant DNA technique. For instance, the enzyme can be extracted in a high yield through a simple treatment such as lysozyme processing or ultrasonic processing by using Escherichia coli as the host microorganism. Also, since the enzyme has thermotolerance, it is possible to remove the enzyme of Escherichia coli participating in the side reaction by subjecting to heat treatment for inactivation and it is also made possible to prepare an enzyme preparation having high specific activity through a convenient operation. In the case of synthesizing a nucleoside analogue using both enzymes including purine nucleoside·phosphorylase and pyrimidine nucleoside·phosphorylase, there may be a phenomenon of excluding efficient reaction due to the rate determination by the reaction in which either one of both enzymes participates. However, purine nucleoside·phosphorylase and pyrimidine nucleoside·phosphorylase can be produced from the thermophilic bacteria only in a constant proportion and it has been extremely difficult to change the proportion. However, it is made possible to carry out efficient synthesis of a nucleoside analogue by separately producing both enzymes through the recombinant DNA technique for modulating the proportion. As

seen herein, the present invention serves in practical application of efficient production of nucleoside analogues so that an extreme benefit in industry is obtained.

[0030]

[Examples]

Specific explanation is given below in relation to nucleoside•phosphorylase derived from Bacillus stearothermophilus TH6-2 strain (deposition in Bi-Ko-Ken, No. 2758) with reference to examples. Also, "Molecular Cloning " (mentioned before) was applied to every item in the examples including preparation of DNA, scission by the restriction enzyme, DNA linking by T4 DNA ligase and transforming process of Escherichia coli. Further, all of various restriction enzymes, T4 DNA ligase, plasmid vectors pUC118 and pUC119, pPstI linker and delattion kit for kilosequence have been obtained from Takara Shuzo K.K.

[0031]

Example 1: Determination of DNA base sequence of DNA molecules containing thermophilic bacteria nucleoside• phosphorylase-structured gene

From the DNA fragment of 4.6 kb coding purine nucleoside•phosphorylase and pyrimidine nucleoside phosphorylase derived from thermophilic bacteria Bacillus stearothermophilus TH6-2 strain previously prepared by Yamanouchi et al. (refer to Figure 1, Japanese Patent Laid-Open Publication Hei 4-4882, Escherichia coli K12 strain retaining pUC119 plasmid in which this fragment is inserted, Escherichia•coli KY-2, has been deposited in Kogyo Gijutsuin (Agency of Industrial Science and

Technology), Biseibutsu Kogyo Gijutsu Kenkyusho (Research Institute for Industrial Technology of Microorganisms) on January 16, 1990 and given with the deposition number "Bi-Ko-Ken Microbe Deposition No. 11197), SacI-EcoRV DNA fragment considered as containing purine nucleoside•phosphorylase-structured gene was sub-cloned to pUC118 and pUC119. The recombinant plasmid was prepared in various clones having distinctive chain length by falling off of a part of the relevant inserted sites using a delation kit for kilosequence following the method of literature (Gene, 28, 351 (1984)). Determination of the DNA base sequence was carried out by the dideoxy chain terminator method (Science, 214, 1295 (1981)) for inserted fragments of various clones thus obtained. Consequently, the DNA sequence of purine nucleoside•phosphorylase-structured gene was obtained as shown in Figure 2. This base sequence is coding a polypeptide of molecular weight 29,637 consisting of 274 amino acids started by Met. The amino acid sequence of 20 amino terminals of this peptide perfectly coincided with that of the refined and isolated purine nucleoside•phosphorylase.

[0032]

Then, with respect to PstI-EcoRI DNA fragment of 2.8 kb considered as containing the pyrimidine nucleoside•phosphorylase-structured gene, pUC118 and pUC119 were subjected to shot gun-subcloning using various restriction enzymes for carrying out determination of the base sequence thereof by the dideoxy chain terminator method similarly to the above. Consequently, the pyrimidine nucleoside•phosphorylase-structured

gene shown in Figure 3 and Figure 4 was obtained. This base sequence is 1,298 bp and is coding a polypeptide of molecular weight 46,271 consisting of 433 amino acids. The amino acid sequence of 10 amino terminals in this peptide perfectly coincided with that of refined and isolated thermophilic bacteria, pyrimidine nucleoside•phosphorylase.

[0033]

Example 2: Preparation of recombinant vector for high expression of thermophilic bacteria nucleoside•phosphorylase

The following method was applied to preparation of recombinant vector pTrc-nunA for producing purine nucleoside• phosphorylase (refer to Figure 5).

That is to say, after scission of a plasmid vector pTrc99A (Gene,69, 301 (1988), obtained from Pharmacia company) using a restriction enzymes NcoI and SmaI, NcoI-HpaI DNA fragment containing purine nucleoside• phosphorylase-structured gene and SD sequence was jointed with the above broken fragment of the plasmid vector pTrc99A using T4 DNA ligase and then Escherichia coli JM105 strain was subjected to transformation using the solution of above scission reaction. The above obtained ampicillin-resistant transformant gave a recombinant vector Trc-punA in which an SD sequence and purine nucleoside• phosphorylase-structured gene were inserted directly behind the trc promoter for expression of the pTc99A.

[0034]

Then, a recombinant vector pTrc-pyn for high expression of pyrimidine nucleoside•phosphorylase was prepared (refer to Figure 6). That is, the above DNA fragment of 4.6 kb

was partly decomposed using a restriction enzyme HincII and the product thereof was further linked with pPstI linker using T4 DNA ligase. The reaction product was further broken with a restriction enzyme PstI for preparing PstI DNA fragment of 2.2 kb containing pyrimidine nucleoside•phosphorylase-structured gene and SD sequence thereof. This DNA fragment and pTrc99A broken with the restriction enzyme PstI were subjected to linking reaction using T4 DNA ligase and further Escherichia coli JM105 strain was subjected to transformation using the above reaction solution. The obtained ampicillin tolerant transformant gave a recombinant vector pTrc-pyn in which the SD sequence and pyrimidine nucleoside• phosphorylase-structured gene were inserted in the scission site directly behind the trc promoter of pTrc99A coinciding with the transcribing direction from trc promoter.

[0035]

Further, a vector pTrc-NE for producing both enzymes of thermophilic bacteria, purine nucleoside•phosphorylase and pyrimidine nucleoside•phosphorylase, was prepared (refer to Figure 7). That is to say, from the above DNA fragment of 4.6 kb, NcoI-EcoRI DNA fragment containing structural genes of both enzymes and the respective SD sequences thereof was linked with Trc99A broken by the restriction enzymes NcoI and EcoRI using T4 DNA ligase and Escherichia coli JM109 strain was transformed using the solution of linking reaction. The obtained ampicillin-tolerant transformant gave a recombinant vector pTrc-NE in which the SD sequences and the structural genes of purine nucleoside• phosphorylase and of pyrimidine nucleoside•

phosphorylase were inserted at the NcoI-EcoRI scission site directly behind trc promoter of pTrc99A.

[0036]

Example 3: Cultivation of transformant and extraction of enzyme

A transformant of Escherichia coli retaining the above 3 kinds of recombinant vectors was planted in 100 ml of 2xYT medium containing 100 μg/ml of ampicillin to be subjected to shake culture at 37 °C. At the time when 4 x 10^8/ml was attained, IPTG was added to the culture medium to obtain 1 mM of the end concentration and shake culture was further continued for 5 hours at 37 °C. After the end of cultivation, the cultivated microbe biomass was collected by centrifugation (9,000 g, for 10 minutes) and suspended in 20 ml of a buffer solution (50 mM EDTA tris hydrochloric acid (pH 7.8), 5 mM EDTA, 0.1 % triton X100). Lysozyme was added to the biomass suspension so as to obtain 1 mg/ml of the end concentration to be incubated at 37 °C for 1 hour to effect bacteriolysis of the transformant with subsequent centrifugation (2,000 g, for 10 minutes) for removal of the biomass residue. The supernatant fraction thus obtained was used as the biomass extract solution. The table below shoes the nucleoside•phosphorylase activity in the biomass extract solution together with those of the control bacteria (Escherichia coli JM105 retaining pTrc99A, and Escherichia coli JM105 containing pUC119-PYR2 (Japanese Patent Laid-Open Publication Hei 4-4882)). The activity of purine nucleoside•phosphorylase and of pyrimidine•nucleoside phosphorylase was calculated on the basis of determination of phosphorolyis activity of respective inosine and uridine at 70

°C carried out following Yamanouchi's method (Japanese Patent Laid-Open Publication Hei 4-4882).

[0037]

[Table 1]

bacteria/ plasmid	purine nucleoside decomposing activity (units/mg protein)	pyrimidine nucleoside decomposing activity (units/mg ptotein)**
JM105/pTrc99A	0.9	1.1
JM105/pUC119•PYR2	17.1	11.6
JM105/pTrc-punA	134.3	(not determined)
JM105/pTrc-pyn	(not determined)	100.3
JM109/pTrc-NE	134.7	66.0

*1 unit = 1 μ mole Hypoxanthine-production/min. at 70 °C

**1 unit = 1 μ mole Uracil-production/min. at 70 °C

[0038]

As shown in Table 1, identification has been attained with respect to the prepared transformant retaining the recombinant vector for the nucleoside phosphorylase activity having 100 fold or more of that of control bacteria (Escherichia coli JM105 retaining pTrc99A) at high temperatures. Also,it has been ascertained that the transformant (bacteria retaining pTrc-NE) prepared in the present invention can produce 6～8 fold of nucleoside•phosphorylase as compared with the transformant (bacteria retraining pUC119-PYR2) prepared by the prior art method (Japanese Patent Laid-Open Publication Hei 4-4882).

Further, the productivity of this transformant corresponds to about 8 fold of that of the thermophilic bacteria Bacillus stearothermophilus TH6-2 strain which is the original strain. Identical property with that of the enzyme derived from thermophilic bacteria was observed for the nucleoside• phosphorylase prepared from the transformant of Escherichia coli retaining these recombinant vectors.

[0039]

Example 4: Synthesis of nucleoside analogue (ribavirin) using cultured microbe biomass

A cultured biomass was collected from 10 ml of culture medium of Escherichia coli JM109 strain retaining pTrc-NE obtained by the method similar to that in Example 3 by centrifugation and was suspended in 1 ml of physiological saline. To this biomass suspension, 40 mM 1,2,4-triazole-3-carboxamide (hereinafter abbreviated as "triazole") as a base donor and 9 ml of 40 mM phosphoric acid buffer solution (pH 6.0) containing 60 mM of uridine as a sugar residue donor were added to be reacted at 45 °C for 1 hour for synthesis of ribavirin. The generated ribavirin was subjected to determination of the yield of ribavirin by HPLC following the method taught in a document (International Patent Laid-Open Publication WO90/10080) giving a result that ribavirin was synthesized in an amount of 92 % as the ratio to triazole. Generation of any byproduct was not observed.

[0040]

Example 5: Synthesis of nucleoside analogue (ribavirin) using culture medium of microbe biomass

To 10 ml of phosphoric acid buffer solution of the constitution identical with that in Example 4 containing triazole uridine, 186 μl of the biomass extract medium derived from the transformant JM109/pTrc-NE prepared in Example 3 was added (end concentration; purine nucleoside•phosphorylase 10 unit/ml; pyrimidine nucleoside•phosphorylase 5 unit/ml) to be subjected to a reaction at 50 °C for 8 hours. The yield of ribavirin was determined by the method similar to that in Example 4 giving a confirmation that ribavirin was synthesized in an amount of 90 % as the ratio to triazole. Generation of any byproduct was not observed similarly to the above.

[0041]

[Brief explanation of the drawing]

[Figure 1]

Figure 1 is illustrating the restriction enzyme map of 4.8 kb SacI-EcoRI DNA fragment containing purine nucleoside•phosphorylase- and pyrimidine nucleoside•phosphorylase-structured gene derived from thermophilic bacteria Bacillus stearothermophilus TH6-1 strain.

[Figure 2]

Figure 2 is illustrating the base sequence of DNA fragment containing purine nucleoside•phosphorylase derived from thermophilic bacteria Bacillus stearothermophilus TH6-1 strain. In the figure, S.D. indicates the SD sequence, Met indicates the codon for translation beginning of purine nucleoside•phosphorylase-structured gene, "stop" indicates the stopping codon thereof, and "Met of qppyn" indicates the codon for translation beginning of pyrimidine nucleoside•phosphorylase-

structured gene, respectively.

[Figure 3]

Figure 3 is illustrating the base sequence of DNA fragment containing pyrimidine nucleoside•phosphorylase-structured gene derived from thermophilic bacteria Bacillus stearothermophilus TH6-1 strain. In the figure, S.D. indicates the SD sequence, Met indicates the codon for translation beginning of pyrimidine nucleoside•phosphorylase-structured gene, and "stop of qppunA" indicates the ending position of purine nucleoside•phosphorylase-structured gene, respectively.

[Figure 4]

Figure 4 is illustrating the base sequence of DNA fragment containing pyrimidine nucleoside•phosphorylase-structured gene derived from thermophilic bacteria Bacillus stearothermophilus TH6-1 strain. In the figure, "stop" indicates the stopping codon ~~thereof~~ thereof. The base sequences shown in Figure 3 and Figure 4 are a series of continued base sequence.

[Figure 5]

Figure 5 is illustrating the constructing method for the recombinant plasmid vector pTrc-punA.

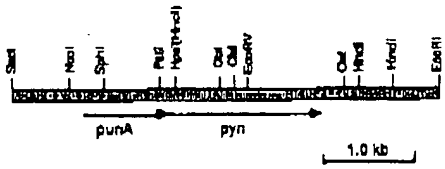
[Figure 6]

Figure 6 is illustrating the constructing method for the recombinant plasmid vector pTrc-pyn.

[Figure 7]

Figure 7 is illustrating the constructing method of the recombinant plasmid vector pTrc-NE.

[Figure 1]



[Figure 2]
【図2】

```
        10         20         30         40         50         60
CCATGGAAAC GACCCTGTTC ATCACGGCAC AGATCATACA CGCCAATACC TACCGCTTCT
NcoI
        70         80         90        100        110        120
TGTGTATAGC CCAAGCATGA ACGGAGGTAA ACAACTTCCA TTGCGTGAAA CCTTTGCACA

       130        140        150        160        170        180
TGTAGGTGCA ACGATTGCAG AAAACTTTGC TGTGAAGATG CCAAAATACG GAACAAGTTT

       190        200        210        220        230        240
TTTACAAGAG TTGAAATAAG GAGCGTGAAC ATATGAATCG AACAGCTATT GAACAACCGC
                        S.D.         Met
       250        260        270        280        290        300
CACAATTTTT AAAAGAAAAG TTTCCAACTT CACCGCAAAT CGGCTTAATT CTTGGCTCTC

       310        320        330        340        350        360
GCTTAGGTGT GTTGGCCGAT GAGATTGAAC AAGCCATTAA AATTCCGTAC AGCGACATTC

       370        380        390        400        410        420
CGAATTTCCC TGTATCGACG GTTGAAGGGC ATGCCGGTCA GCTCGTATAC GGTCAGTTAG

       430        440        450        460        470        480
AAGGGGCAAC AGTAGTCGTT ATGCAAGGGC GGTTTCATTA TTACGAAGGA TACAGCTTCG

       490        500        510        520        530        540
ATAAGGTAAC GTTCCCTGTG CGCGTGATGA AAGCTCTCGG TGTCGAGCAG TTAATTGTCA

       550        560        570        580        590        600
CAAATGCGGC AGGTGGTGTA AATGAATCGT TTGAACCGGG CGATTTAATG ATTATTTCAG

       610        620        630        640        650        660
ATCATATTAA TAACATGGGC GGCAATCCGC TTATCGGTCC GAATGATTCT GCACTTGGGG

       670        680        690        700        710        720
TCCGCTTCCC AGACATGTCG GAAGCATATA GTAAACGACT TCGTCAACTT GCCAAAGATG

       730        740        750        760        770        780
TAGCAAACGA CATCGGTTTA CGTGTGCGCG AAGGTGTGTA TGTCGCCAAT ACGGGTCCAC

       790        800        810        820        830        840
CGTATGAAAC GCCGGCAGAA ATTCGTATGA TTCGTGTCAT CGGTCGCGAT GCTGTTGGTA

       850        860        870        880        890        900
TGTCAACGGT GCCTGAAGTG ATCGTTGCCC GCCATCCCGG AATGGAAGTG CTCGGTATTT

       910        920        930        940        950        960
CGTGTATTTC GAATATGGCT GCAGGTATTT TAGATCAGCC GCTTACGCAT GATCAACTGA

       970        980        990       1000       1010       1020
TCCAAACGAC GGAAAAAGTA AAAGCTGACT TTTTACGATT TCTGAAGGCG ATCGTACCCA

      1030       1040       1050       1060       1070       1080
ACATCGCCAA AAATTAAACG AGAAGGTGAA CGACGATGAG AATGGTCCAT TTAATTGAGA
               Stop                    Met of gppyn
      1090       1100       1110
AAAAACCTGA TGGTCATGCG TTAAC
                        HpaI
```

(Figure3)
【図3】

```
        10         20         30         40         50         60
CTGCACGTAT TTTAGATCAC CCGCTTACGC ATCATGAACT GATCGAAACG ACGGAAAAAC
 PstI
        70         80         90        100        110        120
TAAAAGCTGA CTTTTTACGA TTTGTGAAGG CGATCGTACG CAACATGGCG AAAAATTAAA
                                                   stop of gppunA
       130        140        150        160        170        180
CGAGAAGGTG AACGACGATG AGAATGGTCG ATTTAATTGA GAAAAAACCT GATGGTCATC
    S.D.          Met
       190        200        210        220        230        240
CGTTAACGAA AGAAGAAATT CAGTTTATTA TTCAAGCTTA CACAAAAGGC GATATTCCTG

       250        260        270        280        290        300
ATTATCAAAT GAGCGCATTA GCGATGGCGA TTTTTTTCCC CGGCATGAAT GAAGAAGAGA

       310        320        330        340        350        360
CAGCGGAATT GACGATGGCG ATGGTGCATT CAGGCGATAC GATCGACCTT TCGCCAATTG

       370        380        390        400        410        420
AAGGAATTAA AGTAGACAAA CATTCAACGG GCGGAGTGGG CGATACAACA ACGTTAGTGC

       430        440        450        460        470        480
TTGGCCCTCT TGTCGCCTCC GTCGGTGTTC CGGTTGCGAA AATGTCTGGG CGCGGCCTTG

       490        500        510        520        530        540
GACATACGGG TGGAACGATC GACAAACTAG AATCGGTGCC AGGTTTTCAC GTTGAAATTA

       550        560        570        580        590        600
CGAACGATGA ATTTATCGAT CTTGTCAATA AAAATAAAAT TGCCGTTGTC GGTCAGTCTG

       610        620        630        640        650        660
GTAATTTGAC GCCAGCGGAC AAAAAGTTGT ATGCGCTTCG TGATGTGACG GCAACGGTCA

       670        680        690        700        710        720
ATAGCATTCC GTTAATTGCC TCATCGATTA TGAGCAAAAA AATTGCCCCA GGGGCAGATG

       730        740        750        760        770        780
CGATCGTACT TGACGTAAAA ACAGGTGTGG GCGCGTTTAT GAAACATTTA AACGATGCAA

       790        800        810        820        830        840
AAGCATTAGC GAAAGCGATG GTCGATATCG GAAATCGGCT TGGCCGTAAA ACGATCGCAA

       850        860        870        880        890        900
TTATTTCTGA TATGAGCCAG CCGCTTGCTT ATGCCATTCG AAATGCCCTT GAACTGAAAG

       910        920        930        940        950        960
AAGCGATTGA TACGTTAAAA GGAGAAGGTC CAGAAGATTT CCAAGAGCTG TGCTTAGTGC

       970        980        990       1000       1010       1020
TTGGTAGCCA CATGGTATAT TTAGCGGAAA AAGCATCTTC GCTTGAAGAA GCTCCTCATA

      1030       1040       1050       1060       1070       1080
TGTTAGAAAA AGCGATGAAA GACGGTTCAG CCCTTCAAAC ATTTAAAACG TTCTTAGCTG
```

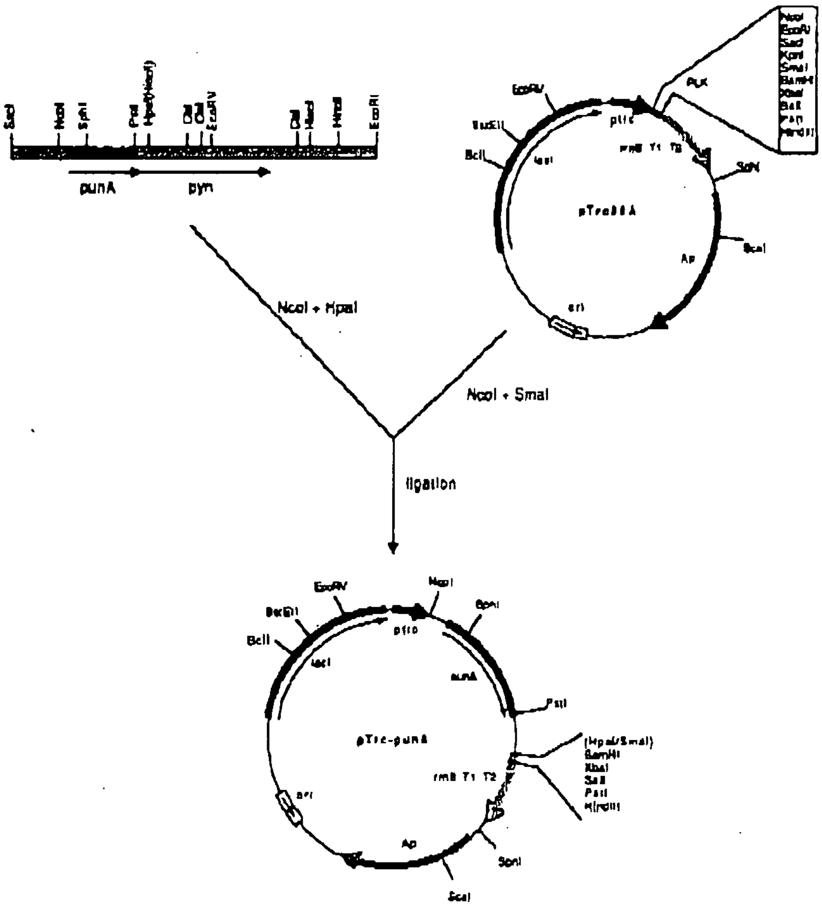
（その1）

〔Figure 4〕〔図4〕

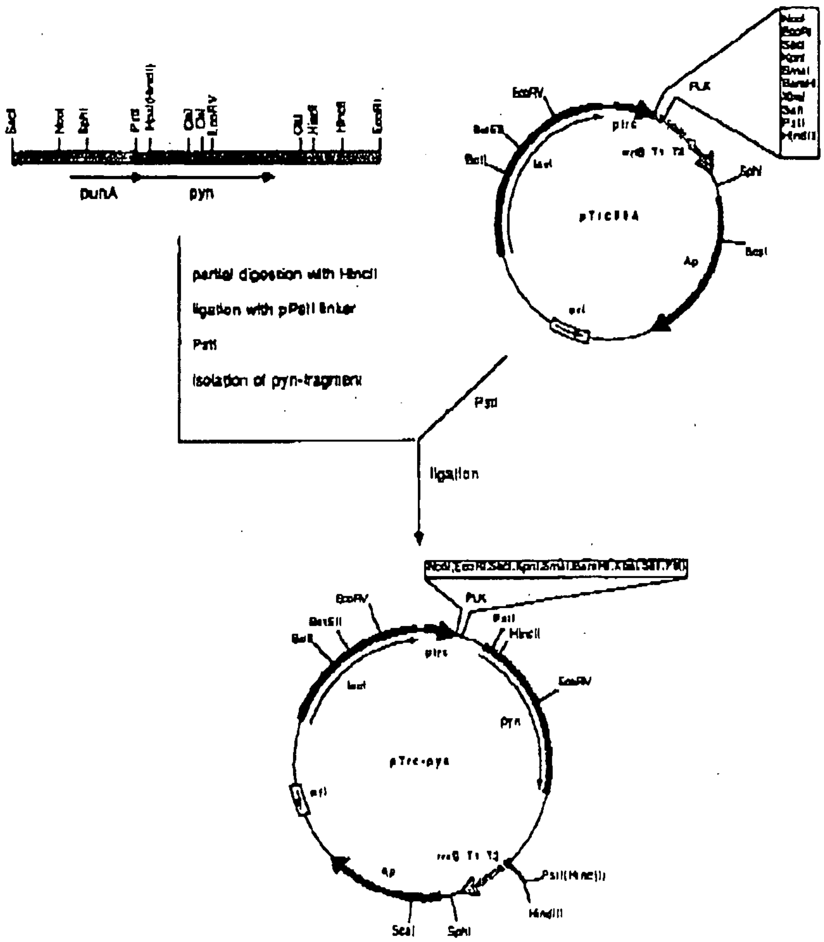
```
      1090       1100       1110       1120       1130       1140
CGCAAGGTGG CGATGCATCT GTTGTCGATG ACCCAAGCAA ATTGCCGCAA GCAAAATATA

      1150       1160       1170       1180       1190       1200
TTATTGAACT AGAAGCGAAA GAAGATGGAT ACGTATCCGA AATTGTCGCG GATGCGGTCG

      1210       1220       1230       1240       1250       1260
GAACGGCGGC GATGTGGCTT GGTGCAGGGC GAGCGACGAA AGAATCAACG ATCGATTTAG

      1270       1280       1290       1300       1310       1320
CTGTCGGTCT CGTGCTTCGC AAAAAAGTCG GCGATGCGGT GAAAAAAGGT GAATCGCTCC

      1330       1340       1350       1360       1370       1380
TTACAATTTA CAGCAACCGT GAACAAGTGG ATGATGTAAA ACAAAAACTA TATGAAAACA

      1390       1400       1410       1420       1430       1440
TTCGTATTTC AGCAACACCT GTTCAAGCTC CAACATTAAT TTACGATAAA ATTTCGTAAC
                                                             stop
      1450       1460       1470       1480       1490       1500
CTGAAGGATT CATTCCTTCA GGTTTTTTTA TGTATAAAAA AATAAAAAAT GGAAAGGATG

      1510       1520       1530       1540       1550       1560
GCAAGTATAA AGAAATGGAG GGTTGGAGAT GAAGCGATTT TTTTGCATGT TTGCTATCGC

      1570       1580       1590       1600       1610       1620
GTTTCTTTTT CTTCCAAGCG TCGTTGTGCG CGCGGAACAG CCGAAAATTG AATTAGCACC

      1630       1640       1650       1660       1670       1680
TGAGGCGCGA TCAGCAATTT TAATTGAGAG AGACACAGGG GCTGTTTTGT ATGAAAAAAA

      1690       1700       1710       1720       1730       1740
TGCCCATGAG CCGCTTCCAC CAGCGAGCAT GACGAAAATT ATGACAATGC TTCTCATTAT

      1750       1760       1770       1780       1790       1800
GGAAGCGATT GATCAAGGAA AGTTGAAGAT AGACGAGCGA GTGCGGGCAA GTGAATACCC

      1810
TGCATCGAT
     ClaI
```

(その2)

(Figure5)
【図5】


SacI
NcoI
SphI
PstI
punA
pyn
EcoRI
EcoRV
BclI
ptrc
lacI
rrnB T1 T2
Ap
ori
NcoI + HpaI
NcoI + SmaI
ligation
EcoRV
BclI
NcoI
ptrc
lacI
PstI
pTrc-punA
(HpaI/SmaI)
BamHI
XbaI
SalI
PstI
HindIII
rrnB T1 T2
ori
Ap
ScaI

[figure6]
【図6】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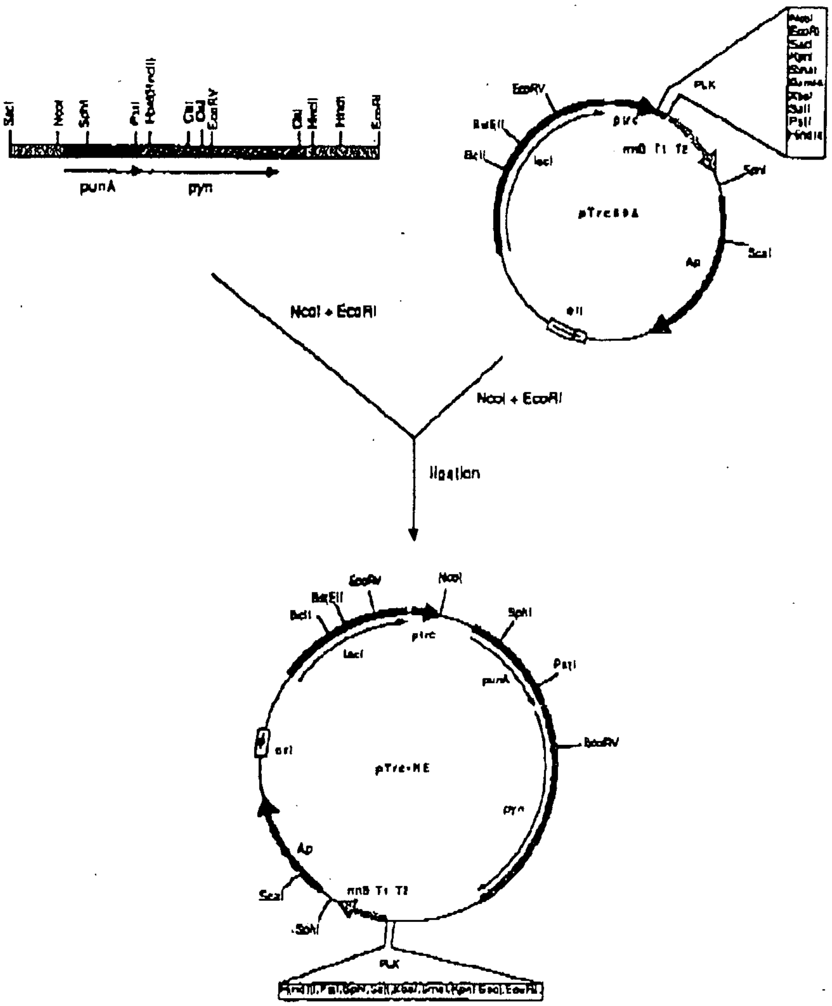
NcoI
SphI
PstI
HpaI(HincII)
ClaI
EcoRV
HincII
EcoRI
punA
pyn
partial digestion with HincII
ligation with pPstI linker
PstI
isolation of pyn-fragment
EcoRV
PLK
ptrc
SphI
ScaI
Ap
ori
lacI
BclI
pTrc99A
PstI
ligation
NcoI,EcoRI,SacI,KpnI,SmaI,BamHI,XbaI,SalI,PstI
HincII
pyn
pTrc-pyn
PstI(HincII)
HindIII
ScaI
SphI

[figure 7]

【図7】



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(51)Int.Cl.[6]		識別記号	庁内整理番号	F I	技術表示箇所
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C12R	1:07)				
(C12N	1/21				
C12R	1:19)				
(C12N	9/10				
C12R	1:19)				